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METHOD FOR ESTIMATION OF BIOTIN IN FOODSTUFFS

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METHOD FOR ESTIMATION OF BIOTIN IN FOODSTUFFS

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Indian Standard

METHOD FOR ESTIMATION OF BIOTIN IN FOODSTUFFS

0. FOREWORD

0.1 This Indian Standard was adopted by the Indian Standards Institution on 15 May 1981, after the draft finalized by the Food Hygiene, Sampling and Analysis Sectional Committee had been approved by the Agricultural and Food Products Division Council.

0.2 Vitamins are required to be assessed in a large number of foodstuffs, such as processed cereals, dairy products, animal feeds, and other natural or manufactured foodstuffs. Moreover, different methods of vitamin assays are used in different laboratories. Therefore, with a view to establishing uniform procedures and also for facilitating a comparative study of results, a series of standards on vitamin assays is being brought out. These would include chemical as well as microbiological methods, where applicable.

0.3 In the preparation of this standard, considerable assistance has been derived from a number of books and publications. However, the method included in this standard is predominantly the one which has been tried in various laboratories in the country, and is mainly based on practical experience gained within the country.

0.4 In reporting the results of a test or analysis, made in accordance with this standard, if the final value, observed or calculated, is to be rounded off, it shall be done in accordance with IS : 2-1960*.

1. SCOPE

1.1 This standard prescribes a microbiological method for the estimation of biotin in foodstuffs.

2. PRINCIPLE

2.1 The *Lactobacillus arabinosus* has a specific requirement for biotin for its growth. The growth response on a defined medium complete in all respects, except the biotin under test, is proportional to the concentration of the biotin added to the medium up to a certain range. The acidity or turbidity produced by the organism is measured to determine the extent of growth and thereby the amount of biotin.

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*Rules for rounding off numerical values (*revised*).

3. APPARATUS

3.1 Autoburette — 5-ml capacity.

3.2 Burettes — 25- and 50-ml capacity.

3.3 Hypodermic Syringe

3.4 Autoclave

3.5 Centrifuge

3.6 Refrigerator

3.7 Inoculating Needle and Loop

3.8 Beakers — 100- and 600-ml capacity.

3.9 Pipettes — graduated, 5 ml and 10 ml.

3.10 Colorimeter

3.11 Conical Flasks — 50- and 125-ml capacity.

3.12 Volumetric Flasks — 100- , 250- and 500-ml capacity.

3.13 Incubator

3.14 Erlenmeyer Flask — 250-ml capacity.

4. REAGENTS AND MEDIA

4.1 Quality of Reagents — Unless specified otherwise, pure chemicals and distilled water (*see* IS : 1070-1977*) shall be employed in tests.

NOTE — 'Pure chemicals' shall mean chemicals that do not contain impurities which affect the test results.

4.2 Acid-Hydrolyzed Casein — conforming to IS : 7203-1974† and free from biotin.

4.3 Cystine-Tryptophan Solution — Suspend 4·0 g of L-cystine and 1·0 g of L-tryptophan in 700 to 800 ml of water, heat to 70 to 80°C and add 20 percent hydrochloric acid dropwise with stirring until the solids are dissolved. Approximately 12 ml of 20 percent hydrochloric acid may be required. Cool to room temperature and make up the volume with water to one litre.

4.4 Adenine-Guanine Uracil (AGU) Solution — Heat 0·1 g each of adenine sulphate, guanine hydrochloride, and uracil in a 250-ml Erlenmeyer flask containing about 75 ml of water and 2 ml of concentrated hydrochloric acid. When the solids have gone into the solution, cool. If a precipitate forms, add a few drops of concentrated hydrochloric acid and heat. Repeat until no precipitate forms on cooling and then transfer to a 100-ml flask and make up the volume with water.

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*Specification for water for general laboratory use (*second revision*).

†Specification for casein hydrolysate (acid digested), microbiological grade.

4.5 Vitamin Solution — Weigh 20 mg of riboflavin, 10 mg of thiamine hydrochloride, 10 mg of *p*-aminobenzoic acid and 40 mg of pyridoxine hydrochloride. Transfer to a one-litre flask and make up the volume with 0·02 N acetic acid.

4.6 Pantothenic Acid Stock Solution — Weigh 54·4 mg of Ca-pantothenate and transfer to a 500-ml volumetric flask. Dilute to the mark with 50 percent ethanol. Store in a refrigerator. The solution contains 100 μg per millilitre of pantothenic acid.

4.7 Biotin Stock Solution — Weigh 25 mg of anhydrous *d*-biotin (free-acid form) and dissolve in 500 ml of 50 percent ethanol.

4.8 Niacin Stock Solution — Dissolve 50 mg niacin in 500 ml of 50 percent ethanol in water.

4.9 Salt Solution A — Dissolve 25 g of dibasic potassium phosphate (KH_2PO_4) and 25 g of monobasic potassium phosphate (K_2HPO_4) in 500 ml of water. Store under toluene.

4.10 Salt Solution B — Dissolve the following ingredients in 500 ml of water :

Magnesium sulphate ($MgSO_4$)	10·0 g
Sodium chloride (NaCl)	0·5 g
Ferrous sulphate ($FeSO_4.7H_2O$)	0·5 g
Manganese sulphate ($MnSO_4.4H_2O$)	0·5 g

Add 5 drops of concentrated hydrochloric acid and store under toluene. Sodium chloride may be omitted when hydrochloric acid-hydrolysed casein is used.

4.11 Anhydrous Glucose

4.12 Anhydrous Sodium Acetate

4.13 Basal Medium for Biotin Assay — To make basal medium for 100 assay tubes, mix the following ingredients in a 600-ml beaker:

Casein hydrolysate	50 ml
Cystine — Tryptophan	50 ml
Adenine — guanine uracil	10 ml
Vitamin solution	10 ml
Salt solution A	10 ml
Salt solution B	10 ml
Anhydrous glucose	10 g
Anhydrous sodium acetate	10 g
OR	
Sodium acetate ($CH_3COONa, 3H_2O$)	17 g
Pantothenic acid stock solution	1 ml
Niacin stock solution	1 ml

4.13.1 Mix the ingredients thoroughly by adding about 250 ml water and then adjust the *p*H to 6·8 with 40 percent sodium hydroxide using a *p*H meter. Transfer to 500-ml volumetric flask and make to volume with water.

4.14 Composition of Agar Medium for Stock Culture

4.14.0 Two media are given for preparation of stock culture tube. Either of these can be used.

4.14.1 Dissolve 5·0 g of yeast-extract (*see* IS : 7004-1973*) in 200 ml of water, and add 1·0 g of anhydrous glucose, 1·0 g of anhydrous sodium acetate and 3 g of agar (*see* IS : 6850-1973†) and heat the mixture on a steam-bath with occasional stirring until the ingredients are dissolved. While the solution is hot, filter it through cotton or cloth and distribute 10 ml to each of 20 culture tubes. Plug with cotton and autoclave for 15 minutes at 120°C.

4.14.2 Dissolve the following ingredients in 200 ml of distilled water, adjust the *p*H to 6·8 and make the volume up to 250 ml:

Peptone (*see* IS : 6853-1973‡)	5·0 g
Yeast-extract (*see* IS : 7004-1973*)	1·0 g
Glucose, anhydrous	10·0 g
Sodium acetate ($CH_3COONa,3H_2O$)	17·0 g
OR	
Sodium acetate, anhydrous	10·0 g
Salt solution A	2·5 ml
Salt solution B	2·5 ml

4.14.2.1 Dissolve 7·5 g of agar (*see* IS : 6850-1973†) in 250 ml of distilled water with the aid of heat and mix the solution well with the above solution. While the solution is still hot, take 10 ml of solution into test tubes. Plug with cotton, and autoclave for 15 minutes at 120°C. After cooling to room temperature store the tubes at 2 to 4°C and use for maintaining stock cultures.

4.15 Preparation of Inoculum Broth — Prepare the inoculum broth in the same way as the culture medium except that instead of agar solution, use more of distilled water (after adjusting the *p*H to 6·8) to make up the volume to 500 ml. Take 10 ml of the solution into test tubes, then plug with cotton, autoclave for 15 minutes at 120°C, cool to room temperature and store at 2 to 4°C.

---

*Specification for yeast extract, microbiological grade.

†Specification for agar, microbiological grade.

‡Specification for peptone, microbiological grade.

4.16 Isotonic Salt Solution — Weigh 0·9 g of sodium chloride and transfer to a 100-ml flask and make up the volume. Transfer 10 ml quantities of this solution to culture tubes, and autoclave as given in **4.14**.

4.17 Sodium Hydroxide — 1·0 and 0·1 N.

4.18 Bromothymol Blue Indicator Solution — Dissolve 0·1 g of bromothymol blue in 1·6 ml of 0·1 N sodium hydroxide and triburate until the powder disappears. Dilute to 250 ml. The dye may also be dissolved in 95 percent ethanol and diluted with 0·1 N sodium hydroxide (1·6 ml) followed by making up the volume with water.

4.19 Sulphuric Acid (6N) — Prepare 9 litres of 6 N sulphuric acid by adding 1 500 ml of concentrated sulphuric acid slowly and with stirring to about 7 litres of water. After cooling make to 9 litres with water.

4.20 Sodium Hydroxide Solution (20 Percent) — To 40 g of sodium hydroxide pellets add 160 ml of water and stir until it dissolves. The solution may be used to neutralize the acid used in the extraction of the samples.

4.21 Working Biotin Standard — Dilute 5 ml of the biotin stock solution to 250 ml with 50 percent ethanol. Dilute 5 to 500 ml with 50 percent ethanol. Prepare the working standard every day by diluting 5 ml of diluted solution to 250 ml with water. This working standard contains 0·2 mμg (ng) per millilitre.

5. PROCEDURE

5.1 Preparation and Maintenance of Stock Culture of *Lactobacillus arabinosus* — Inoculate pure culture of *Lactobacillus arabinosus* 17-5 (ATCC 8014) to the sterile agar culture tube prepared as described in **4.14**. Inoculate the tube for 16 to 24 hours at 37°C and store at 4°C. Transfer the culture into new agar tube every fortnight.

5.2 Preparation of Inoculum — A day prior to use inoculate, inoculum broth tube with culture from the stock culture and incubate at 37°C for 16 to 18 hours. Use a centrifuge tube plugged with cotton, an all glass syringe, a 0·8-mm needle, some saline (0·9 percent) in a conical flask. Sterilize saline by autoclaving for 15 minutes at 120°C and syringe, needle and centrifuge tube by heat in a hot air oven at 160°C for one hour. Transfer the cells from the inoculum tube to the centrifuge tube and centrifuge.

Decant the supernatant and re-suspend the cells in sterile saline and centrifuge again. Repeat the process two or three times. Again make the washed cells into a suspension using the saline. Take re-suspended cells in the sterile syringe and inoculate the assay tubes with one drop each of the inoculum. The optical density of the inoculum should be within the range of 0·1 to 0·12.

5.3 Preparation of Samples

5.3.1 Weigh or pipette sufficient material to contain approximately 200 mμg (ng) of biotin into a 50-ml conical flask. Add 25 ml of 6 N sulphuric acid and thoroughly mix. Autoclave the mixture at 120°C for two hours. Cool, transfer to a 100-ml volumetric flask, make to volume, and filter if necessary.

NOTE — In choosing the quantity of material to be extracted, the degree of homogenety with respect to biotin should be considered. Samples smaller than 1 to 2 g should be avoided, if possible. For high potency samples (that is liver), the samples may need to be diluted considerably to permit assay of the biotin content.

5.3.2 Dilute a 10-ml aliquot to 60 to 70 ml with water in a 100-ml beaker and neutralize with 20 percent sodium hydroxide to *p*H 6·8 (*p*H meter or bromothymol blue indicator). Dilute to 100 ml in a volumetric flask.

NOTE 1 — If the quantity of biotin in the original samples differs widely from 200 mμg (ng), it will be necessary to dilute accordingly so that the final solution contains approximately 0·2 mμg (ng) per ml.

NOTE 2 — The use of two steps in the dilution procedure rather than diluting originally to one litre permits changes in the final dilutions without having to rehydrolyze the sample in case the potency is much less than expected.

5.4 Preparation of Tubes for Standard Curve

5.4.1 To duplicate tubes add 0·0, 0·5, 1·0, 1·5, 2·0, 2·5, 3·0, 4·0, and 5·0 ml volumes of the working biotin standard solution [0·2 m μg (ng) per ml], and distilled water to a final volume of 5·0 ml in each tube. To each tube add 5·0 ml of the biotin basal medium. Cover with cotton and brown paper and sterilize at 115°C for 10 minutes.

5.5 Preparation of Assay Tubes

5.5.1 To duplicate tube add 1·0-, 2·0- and 3·0-ml aliquots of the test solution.

NOTE 1 — The use of different levels permits evaluation of the validity of the assay over a range of biotin concentration within the limits of the standard curve.

NOTE 2 — Where the approximate potency of the sample is not known, it may be desirable to add the test solution over a greater range of concentration (that is, 0·5, 1·0, 2·0, 3·0, 4·0 and 5·0 ml) where 6 tubes are used for sample.

5.5.2 Add distilled water to a volume of 5·0 ml in each tube. Add 5·0 ml of the biotin basal medium to each tube. Cover the tubes with cotton and brown paper, autoclave at 115°C for 10 minutes and cool to room temperature.

5.6 Inoculation and Incubation — Aseptically inoculate each tube with one drop of inoculum except the blank. Incubate at 37°C for 18 to 20 hours.

5.7 Titrimetric Method

5.7.1 Transfer the contents of each tube to a 125-ml conical flask and rinse the tube once with about 10 ml water, adding the rinsing to the flask. Add about 0·2 ml of 0·1 percent bromothymol blue and titrate with 0·1 N sodium hydroxide, to a green colour, approximate *p*H 6·8. Hold a flask for reference colour for about 10 to 20 titrations and then substitute by a new flask.

5.7.2 *Calculation* — Draw a standard curve for the assay by plotting millilitre of 0·1 N sodium hydroxide used in titration of the standard tubes against mμg (ng) of biotin per tube in the standard series. Determine the biotin content of the tubes in the unknown series by interpolation of the titre values on the standard curve. Discard any values which show more than 1·00 mμg (ng) or less than 0·1 mμg (ng) biotin per tube. Calculate the biotin content for each of the duplicate sets of tubes. Calculate the biotin content of the test material from the average of the values for each of the solution obtained from not less than 3 sets of these tubes which do not vary by more than 10 percent from the average, using the following formula and express the results as μg per 100 g of sample:

$$\text{m}\mu\text{g (ng) of biotin per g of sample} = \frac{\text{Average m}\mu\text{g} \times \text{volume}}{\text{Mass of sample}} \times \text{dilution factor}$$

5.8 Turbidimetric Method

5.8.1 At the end of the incubation period (*see* **5.6**) remove the assay tubes and steam for 5 minutes. Cool and read turbidimetrically in a colorimeter at 660 mm using the uninoculated blank tube to set the instrument to zero.

5.8.2 *Calculation* — Draw a standard curve for the assay plotting the optical density or turbidity reading on the X-axis against concentration of the biotin on the Y-axis. Determine the biotin content of the tubes in the unknown series by interpolation of the colorimeter readings on the standard curve. Calculate the average for one millilitre of the test solution from values obtained from not less than three sets of tubes which do not vary by more than 10 percent on the average. Calculate the biotin content of the test solution using the following formula and express the results as μg per 100 g of sample:

$$\text{m}\mu\text{g (ng) of biotin/g sample} = \frac{\text{Average m}\mu\text{g/ml} \times \text{dilution factor}}{\text{Mass of the sample}}$$

5.8.3 It is essential that a curve constructed each time in assay is undertaken since it is not possible to duplicate exactly from time to time conditions of autoclaving, temperature to incubation, etc, which influence the standard curve readings.

INDIAN STANDARDS
ON
FOOD ANALYSIS

IS:	
5398-1968	Methods for estimation of thiamine (vitamin B_1) in foodstuffs
5399-1969	Methods for estimation of riboflavin (vitamin B_2) in foodstuffs
5400-1969	Methods for estimation of nicotinic acid (niacin) in foodstuffs
5401-1969	Methods for detection and estimation of coliform bacteria in foodstuffs
5402-1969	Method for plate count of bacteria in foodstuffs
5403-1969	Method for yeast and mould count in foodstuffs
5835-1970	Method for estimation of vitamin D in foodstuffs
5838-1970	Method for estimation of vitamin C in foodstuffs
5886-1970	Methods for estimation of carotenes and vitamin A (retinol) in foodstuffs
5887	Methods for detection of bacteria responsible for food poisoning:
(Part I)-1976	Isolation and identification of enteropathogenic *Escherichia coli* and the enumeration of *Escherichia coli*
(Part II)-1976	Isolation, identification and enumeration of *Stophylococcus aureus* and faecal *Streptococci*
(Part III)-1976	Isolation and identification of *Salmonella* and *Shigella*
(Part IV)-1976	Isolation and identification of *Clostridium welchii*, *Clostridium Botulinum* and *Bacillus cereus* and enumeration of *Clostridium welchii* and *Bacillus cereus*
(Part V)-1976	Isolation, identification and enumeration of *Vibro cholerae* and *Vibro parahaemolyticus*
7219-1973	Method for determination of protein in food and feeds
7234-1974	Method for estimation of folic acid in foodstuffs
7235-1974	Methods for estimation of tocopherol (vitamin E) in foodstuffs
7529-1975	Method for estimation of vitamin B_{12} in foodstuffs
7530-1975	Method for estimation of pyridoxine (vitamin B_6) in foodstuffs
7815-1975	Method for estimation of amino acids in food
8168-1976	Method for determination of available lysine in foods